## REMARKS

Applicants first wish to thank Examiner Kam for granting applicants a telephonic interview on October 29, 2004 to discuss the outstanding office action.

Claims 1-2, 4-11, 13-17, 19, and 23-51 were pending in the subject application. Applicants have amended claims 1, 2, 5-10 and 15 to clarify the claimed subject matter and have canceled claims 45-51 without prejudice to their right to pursue the subject matter of these claims in a later filed application. Applicants have added dependent claims 52-55. Support for claim 52 may be found in originally-filed claim 14 and on page 19, lines 25-26 of the originally-filed specification. Support for claim 53 may be found in originally-filed claim 1 and on page 28, lines 10-14 of the originally-filed specification. Support for claims 54 and 55 may be found in originally-filed claims 9 and 10, respectively. This amendment does not involve any issue of new matter. Applicants respectfully request entry of the subject amendment such that claims 1-2, 4-11, 13-17, 19, 23-44 and 52-55 will be pending.

Applicants will address all issues in the order that they appear in the Office Action.

### Objection to New Matter

The Examiner rejects claims 45-51 under 35 U.S.C. 132 as allegedly introducing new matter into the disclosure. Without conceding the correctness of the Examiner's argument, applicants have cancelled claims 45-51 without prejudice to their right to pursue the subject matter of these claims in a later filed application, thus obviating this ground of rejection. Accordingly, applicants respectfully request reconsideration and withdrawal of this ground of rejection.

### Claim Objections

The Examiner objects to claim 4 under 37 CFR 1.75 as allegedly being a substantial duplicate of claim 2. In response, applicants have amended claim 2 to recite a translation system substantially free of all four transcription factors, thus obviating this ground of rejection. Support for this amendment may be found, *inter alia*, on page 19, lines 29-30, and page 49, lines 2-3. The Examiner

further objects to the term "□-cyanoalanine" in claim 5. In response, applicants have replaced this term with "β-cyanoalanine", as recited in originally-filed claim 5. Accordingly, applicants respectfully request reconsideration and withdrawal of these grounds of rejection.

Claim Rejections 112, 1st Paragraph

The Examiner rejects claims 45-51 under 35 U.S.C. 112, 1st paragraph as allegedly failing to comply with the written description requirement. In response, without conceding the correctness of the Examiner's argument but merely to expedite allowance of the pending claims, applicants have cancelled these claims without prejudice to their right to pursue the subject matter of these claims in a later filed application. Accordingly, applicants respectfully request reconsideration and withdrawal of these ground of rejection.

Claim Rejections 112, 2nd Paragraph

The Examiner rejects claims 8 and 45-51 under 35 U.S.C. 112, 2nd paragraph as allegedly failing to particularly point out and distinctly claim the subject matter that the Examiner regards as their invention. As noted above, applicants have canceled claims 45-51, thus making the rejection with respect to these claims mute. With regards to claim 8, the examiner alleges that there is insufficient antecedent basis for the phrase "said inactive tRNA species". In response, applicants have amended this phrase to recite "said **active** tRNA species", as recited in section (a) of the claim. Accordingly, applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Claim Rejections 35 USC §102

The Examiner rejects claims 7, 19, 24 and 39 under 35 U.S.C. 102(b) as allegedly anticipated by Hohsaka et al. (J. Am. Chem. Soc. 121, 12194-12195 (1999)).

In response, Applicants traverse the Examiner's rejection. Applicants submit that, pursuant to MPEP 2131.01, "a claim is anticipated only if each and every element as set forth in the claim is found, either expressly or inherently described, in a single prior art reference."

Hohsaka, however, fails to set forth every element of claim 7 in accordance with MPEP 2131.01. For example, claim 7 of the subject application recites the following three elements of the cell-free translation system:

(i) lacks one or more active wild-type amino acyl tRNA species,

(ii) lacks the ability to synthesize said wild-type amino acyl tRNA species, and

(iii) the exogenous elongator amino acyl tRNA species replaces the wild-type elongator amino acyl tRNA species.

By contrast to the claimed invention, Hohsaka's translation system fails to set forth, expressly or inherently, each of these three elements.

First, rather than lacking one or more active wild-type amino acyl tRNA species as recited in claim 7, Hohsaka's translation system contains all active wild-type amino acyl tRNA species. Hohsaka's translation system uses a crude *E. coli* extract, as recited on page 12194, column 1, second paragraph: "[t]hen, the tRNAs and mRNAs were added into the *E. coli in vitro* protein synthesizing system.[12]" Reference 12 corresponds to Hohsaka et al., *J. Am. Chem. Soc.* (1999) 121, 34-40 (**Exhibit A**). Reference 12 recites an *E. coli* S30 extract on page 36, 1st column, first full-paragraph the E. coli S30 extract used in Hohsaka: "*In vitro* translation was carried out in a 10μL of a reaction mixture containing 55mM Hepes...0.1 nmol of amino acyl tRNA, and 2μL of *E. coli* S30 extract" (emphasis added).

Secondly, rather than lacking the ability to synthesize said wild-type amino acyl tRNA species, Hohsaka's translation system has the ability to synthesize all wild-type amino acyl tRNA species *i.e.* contains all endogenous amino acyl tRNA synthetases, since it teaches the use of crude *E. coli* S30 extract.

Finally, rather than replacing wild-type elongator amino acyl tRNA species with exogenous elongator l tRNA species, Hohsaka's translation system adds exogenous amino acyl tRNAs to the

full-complement of wild-type elongator amino acyl tRNA species present in the *E. coli* S30 extract. Even the two naturally-occurring arginyl amino-acyl tRNAs which can compete with the exogenous amino acyl tRNAs are present in Hohsaka's system, as set forth on page 12194, column 2, first paragraph:

> If the CGGG codon was undesirably read as a CGG triplet by the endogenous arginyl $tRNA_{CCG}$, the reading frame remained unshifted, resulting in the encounter of the AUG codon that is underlined. Similarly, if the AGGU codon was read by the AGG triplet by the arginyl $tRNA_{CCU}$, the protein synthesis will stop at the UGA stop codon underlined.

In summary, since Hohsaka fails to teach or suggest, either expressly or inherently, the three claim elements cited above, it fails to anticipate claims 7, 19, 24 and 39. Accordingly, applicants respectfully request reconsideration and withdrawal of this ground of rejection.

Applicants further note that the Examiner states on page 7 of the outstanding office action that she has not given patentable weight to the phrase reciting "highly selective incorporation" in claim 7, since she has interpreted the term "capable of" as describing an optional feature of the translation system . Therefore, Applicants have deleted the phrase "highly selective incorporation" from claim 7 and have introduced this language into new claim 53 which depends from claim 7.

Claim Rejections 35 USC §102

The Examiner rejects claims 1, 11, 14, 23, 28, 38, 40, 41 and 44 under 35 U.S.C. 103(a) as allegedly anticipated by Rothschild et al. (U.S. Patent 5,643,772, July 1, 1997).

Applicants traverse the Examiner's rejection. In the Office Action, the Examiner asserts on page 9, last sentence of the first paragraph, that she has not given any patentable weight of the phrase "capable of translating exogenously added mRNA species with highly selective incorporation at each codon to form the peptidomimetic product", even though applicants had submitted on the amendment filed June 14, 2004, that highly selective incorporation was a feature of claim 1. Based on a telephonic interview by the undersigned with the Examiner on October 30, 2004, applicants

have amended claim 1 to recite a translation system "**that translates** exogenously added mRNA species with highly selective incorporation at each codon to form the peptidomimetic product.", as the Examiner indicated that such amendment would give patentable weight to the phrase. Accordingly, Applicants respectfully request that the Examiner give patentable weight to this claim element.

MPEP 706.02(j) sets forth three basic criteria needed to establish a *prima facie* case of obviousness: 1) the prior art references must teach or suggest all the claim limitations; 2) some motivation or suggestion, either found in the references themselves or in the knowledge generally available to one of ordinary skill in the art, to combine or modify the references must be present; and 3) a reasonable expectation of success is required.

Rothschild, however, fails to teach or suggest all the claim elements of claim 1, from which the other rejected claims depend, and thus the *prima facie* obviousness rejection is improper. In particular, applicants submit that Rothschild fails to teach or suggest a translation system with highly selective incorporation (*i.e.* at least 80% incorporation) at each codon of an exogenous mRNA to form a peptidomimetic product.

The examples in Rothschild using wheat germ extracts do not appear to have achieved any highly selective incorporation, and Rothschild does not make any assertions that its system is highly selective. Furthermore, it is unlikely that the crude extracts in the examples would inherently achieve the highly selective incorporation recited in claim 1, since they are crude systems prone to competition between amino acyl tRNAs charged with unnatural amino acids and endogenous wild-type elongator amino-acyl tRNAs.

Rothschild also fails to teach or suggest how highly selective incorporation would be achieved in an *in vitro* cell free system. For instance, while Rothschild states on page 8, lines 7-12 that "[m]ixtures of purified translation factors have also been used successfully to translate mRNA into protein as well as combinations of lysates or lysates supplemented with purified translation

factors such as initiation factor-1 (IF-1), IF-2, IF-3 (α or β), elongation factor T (EF-Tu), or termination factors," Rothschild fails to disclose which combinations of purified factors would be necessary or sufficient to achieve highly selective incorporation. In fact, in the absence of additional factors, the combination of the translation factors recited by Rothschild *i.e.* initiation factor-1 (IF-1), IF-2, IF-3 (α or β), elongation factor T (EF-Tu), and termination factors would not be expected to generate a highly selective translation system. Accordingly, Rothschild fails to enable a translation system with highly selective incorporation of unnatural amino acids or amino acid analogs into a peptidomimetic product.

Applicants draw the Examiner's attention to the following reference: Sergey Mamaev, Jerzy Olejnik, Edyta Krzymanska Olejnika and Kenneth J. Rothschild, (2004) *Analytical Biochemistry* Vol. 326(1), p25-32 (**Exhibit B**, "Mamaev *et al.*"), coauthored by K.J. Rothschild and Jerzy Olejnik, two of the inventors of the Rothschild reference. Published in 2004, Mamaev *et al.* relates to a high efficiency cell-free translation system, as is evident from the abstract:

> A highly efficient method for the introduction of fluorophores and other markers at the N terminus of proteins produced in a cell-free extract has been developed. The method utilizes an amber (CUA) initiator suppressor tRNA chemically aminoacylated with a fluorophore–amino acid conjugate which is introduced into an *Escherichia coli* S30 cell-free translation system.

In characterizing the novel translation system, Mamaev *et al.* further recites in the first paragraph of the conclusion, on page 31, as follows:

> This paper describes a system for labeling proteins at their N-terminal end using cell-free expression and amber suppression. This system offers significant advantages over existing methods for cell-free protein labeling. As discussed above, the labeling efficiency ranged from 27 to 67%, which is a significant improvement over existing methods using elongator or wild-type initiator tRNAs (emphasis added).

Thus, in comparing their cell-free system to the preceding translation systems, Mamaev *et al.* claims that their 27-67% efficiency of labeling is superior to the previous systems that use

elongator tRNAs species. It may be inferred that previous translations systems that use elongator sense tRNAs had efficiencies below 27%. Applicants submit that the examples in Rothschild are based on elongator tRNAs, and thus expected to display this low selectivity.

Since Rothschild fails to teach or suggest all the limitations of the claimed translation system, it fails to anticipate the claimed invention. Applicants request reconsideration and withdrawal of this ground of rejection.

## CONCLUSIONS

In view of the above, each of the presently pending claims in this application is believed to be in immediate condition for allowance. Accordingly, the Examiner is respectfully requested to pass this application to issue.

Applicant believes that no fee is due at this time. However, if any additional fee is due, please charge our Deposit Account No. 18-1945, under Order No. AFOR-P01-001 from which the undersigned is authorized to draw.

Dated: December 8, 2004

Respectfully submitted,

By _____

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*J. Am. Chem. Soc.* **1999**, *121*, 34−40

# Efficient Incorporation of Nonnatural Amino Acids with Large Aromatic Groups into Streptavidin in In Vitro Protein Synthesizing Systems

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**Abstract:** Efficiencies of the incorporation of various nonnatural amino acids carrying aromatic side groups into streptavidin were examined. The aromatic amino acids were linked to a mixed dinucleotide, pdCpA, and the resulting aminoacyl pdCpAs were coupled with tRNAcccg(−CA) to afford chemically aminoacylated tRNAcccg's. Mutant streptavidin mRNA containing a CGGG 4 base codon at the Tyr83 site was prepared and added to an *Escherichia coli* in vitro translation system with the aminoacyl tRNAcccg. The expression of the full-length mutant streptavidins was confirmed by a Western blot analysis, and their biotin binding activity was examined by a dot blot analysis. The Western blot analysis indicated that the efficiencies of the incorporation were higher for aromatic groups with straight configurations than those with widely expanded or bend configurations. The incorporation efficiencies were also examined in a rabbit reticulocyte lysate. In the latter system, the efficiencies were markedly improved for nonnatural amino acids with large side groups such as pyrene and anthraquinone.

Site-specific incorporation of nonnatural amino acids into proteins through in vitro protein biosynthesizing systems is becoming an important technique for structure−function analysis and for artificial functionalization of proteins.[1] By incorporating nonnatural amino acids into proteins, we can introduce a variety of specialty functions depending on the side groups of the amino acids. Particularly, nonnatural amino acids carrying various aromatic groups may serve as fluorescent probes for the analysis of microenvironment of proteins and as electron donors and acceptors for building up pathways for electron transfers in proteins.[2] For this technique to be used widely in the field of protein engineering, however, it is essential to know what type of nonnatural amino acids will be successfully incorporated and what type will be rejected in the *Escherichia coli* and other protein biosynthesizing systems. The nonnatural amino acids that have been examined so far were restricted to those carrying rather small side groups, and the incorporation efficiencies of the nonnatural amino acids with large aromatic groups have not been studied extensively. From a few examples reported so far, however, it is suggested that the efficiency may depend sharply on the side groups. For instance, *p*-benzoylphenylalanine has been successfully incorporated into T4 Lysozyme[3] and cytochrome *b*2[4] in good yield, whereas the incorporation of the amino acid carrying oxyltetramethylpyrroline was unsuccessful.[3] In this study we have explored a relationship between the structure and incorporation efficiency of nonnatural amino acids carrying 19 different side groups in the *E. coli* and rabbit reticulocyte in vitro systems.

Since the amino acid selectivity is mainly governed by the amino acid adaptability of the ribosomal systems[5] and the adaptability depends on the structure of ribosomes, the different ribosomal systems are expected to show different amino acid selectivity. And the results will be useful for designing proteins incorporated with nonnatural amino acids carrying large side groups with specialty functions.

Another important step for the incorporation of nonnatural amino acids is the assignments of codons to the amino acids. Most of the workers have been using nonsense codons for this purpose. But the number of nonsense codons that can be assigned to nonnatural amino acids is limited to be less than two. We have proposed in the previous paper that a four-base codon−anticodon pair, AGGU-ACCU, worked effectively for this purpose in the *E. coli* in vitro system.[6] The frameshift suppressor tRNAaccu's chemically aminoacylated with nonnatural amino acids carrying *p*-nitrophenyl, 1- and 2-naphthyl, 2-anthryl, and *p*-phenylazophenyl groups, respectively, success-

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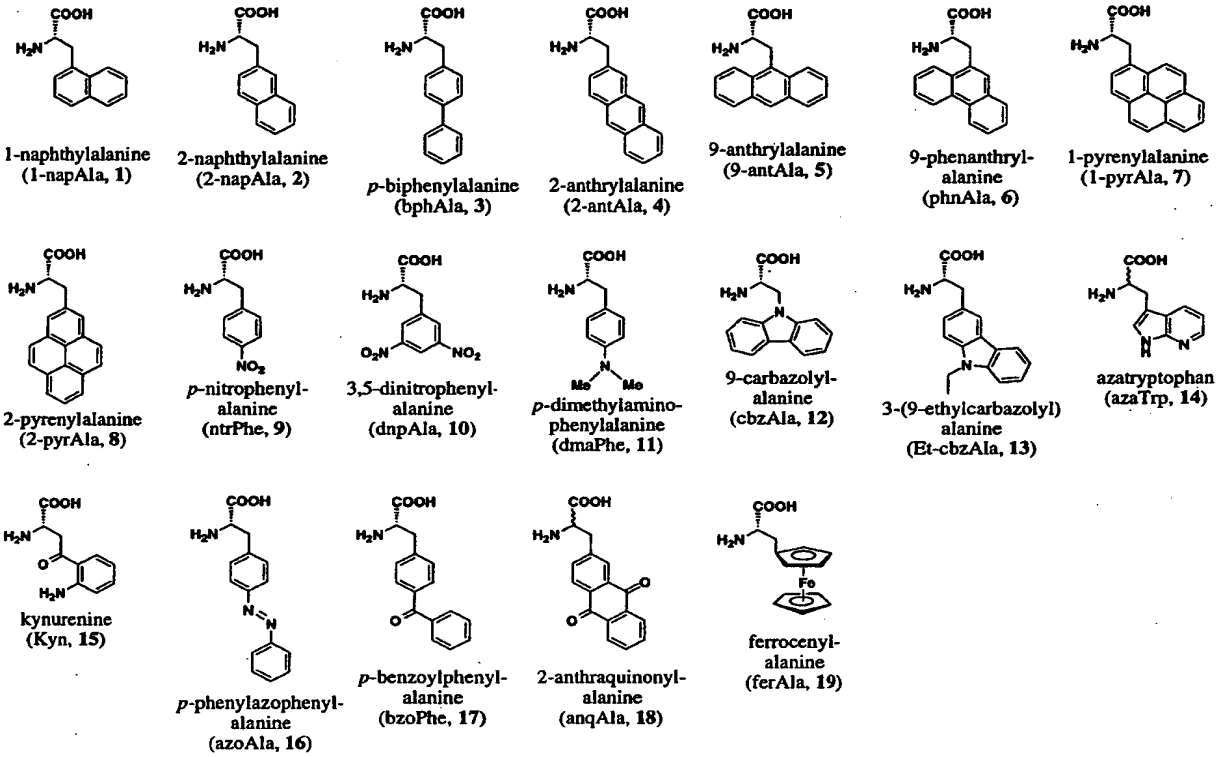


**Figure 1.** Structure of aromatic nonnatural amino acids examined in this study.

fully suppressed the frameshift mutation in a mRNA and produced mutant proteins containing the nonnatural amino acids. In the case of nitrophenylalanine, the suppression efficiency was about 20%.[6] The frameshift strategy using the four-base codon–anticodon pairs will be much more advantageous than the nonsense codon strategy because of the possible extension to incorporate two or more different nonnatural amino acids into single proteins. In this study, we have used another four-base codon–anticodon pair, CGGG-CCCG, because of its higher efficiency than the AGGU-ACCU pair.

Streptavidin was selected as a target protein, since its structure and function have been characterized extensively, and its activity can be easily evaluated by using biotinylated enzymes or biotinylated fluorophores. In this paper, we evaluated incorporation efficiencies of nonnatural amino acids with various aromatic side groups as listed in Figure 1. The aromatic amino acids were of the L-form except for DL-anthraquinonylalanine and DL-azatryptophan. They were incorporated at the Tyr83 site of streptavidin by using a CGGG-CCCG codon–anticodon pair. The incorporation efficiencies were evaluated from the Western blotting of the reaction mixture of the in vitro system.

## Materials and Methods

L-*p*-Nitrophenylalanine, L-1-naphthylalanine, L-2-naphthylalanine, DL-azatryptophan, and L-kynurenine were purchased from Sigma (St. Louis, MO). Boc-L-*p*-benzoylphenylalanine was from Bachem (Switzerland). L-*p*-Biphenylalanine,[7a] L-9-anthrylalanine,[7b] L-9-phenanthrylalanine,[7c] L-1-pyrenylalanine,[7d] L-2-pyrenylalanine, and L-3-(9-ethylcarbazolyl)-alanine[7e] were synthesized from the corresponding arylaldehydes and acetylglycine. L-2-Anthrylalanine, L-3,5-dinitrophenylalanine, DL-2-anthraquinonylalanine,[7f] and L-ferrocenylalanine[7g] were synthesized from the corresponding arylmethyl halides and diethyl acetamidomalonate. L-9-Carbazolylalanine[7h] was synthesized from carbazole and 2,3-dibromopropanoic acid. L-*p*-Dimethylaminophenylalanine[7i] and L-*p*-phenylazophenylalanine[7j] were synthesized from L-*p*-nitrophenylalanine. Of the above 19 amino acids, L-2-anthrylalanine, L-2-pyrenylalanine, and L-3,5-dinitrophenylalanine were newly synthesized in this work. The details of the syntheses of the first two are described in the Supporting Information. DL-3,5-Dinitrophenylalanine was synthesized from 3,5-dinitrobenzyl chloride and diethyl acetamidomalonate and selectively deacetylated with acylase. The detail will be published elsewhere.

Vent DNA Polymerase, T7 RNA polymerase, and Prestained Protein Marker were purchased from New England Biolabs (Beverly, MA). T4 RNA ligase was from Takara Shuzo (Kyoto, Japan). *E. coli* S-30 extract, Rabbit Reticulocyte Lysate Systems, and ProtoBlot II AP System for the Western blot analysis were from Promega (Madison, WI). Plasmid encoding streptavidin was from R&D Systems Europe (Abingdon, U.K.). Immun-Blot PVDF Membrane was from Bio-Rad (Hercules, CA). RNase Inhibitor was from Wako Chemicals (Osaka, Japan). T7-Tag Antibody was from Novagen (Madison, WI). Biotinylated alkaliphosphatase was from Zymed Laboratories (San Francisco, CA). Nitrocellulose membrane was from Toyo Roshi (Tokyo, Japan). Other biochemicals were from Sigma.

**Synthesis of Aminoacyl tRNA.** The syntheses of aminoacyl pdCpAs and tRNA(-CA)s are described in the Supporting Information. The ligation reaction was carried out in a mixture that contained 1 nmol of tRNA(-CA), 20 nmol of aminoacyl pdCpA in DMSO, 1 mM ATP, 15

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mM $MgCl_2$, 3.3 mM DTT, 20 μg/mL of BSA, and 60 units of T4 RNA ligase in a 40 μL of 55 mM Hepes−Na (pH 7.5). The mixture was incubated at 4 °C for 2 h, then diluted with 1 vol of prechilled 0.6 M potassium acetate (pH 4.5). The solution was extracted with phenol/chloroform and chloroform, then the aminoacyl tRNA was precipitated with 3 vol of ethanol. The pellet was dissolved in 4 μL of prechilled 1 mM potassium acetate (pH 4.5), and immediately the solution was added to the reaction mixture of the in vitro translation. The purity of the tRNA was confirmed by a 10% PAGE with 7 M urea.

**In Vitro Protein Biosynthesis of Mutant Streptavidins and Evaluation of the Incorporation Efficiency.** The preparation of mRNA was described in the Supporting Information. In vitro translation was carried out in a 10 μL of a reaction mixture containing 55 mM Hepes−KOH (pH 7.5), 210 mM potassium glutamate, 6.9 mM ammonium acetate, 9 mM magnesium acetate, 1.7 mM DTT, 1.2 mM ATP, 0.28 mM GTP, 26 mM phosphoenolpyruvate, 1 mM spermidine, 1.9% poly(ethylene glycol)-8000, 35 μg/mL folinic acid, 0.1 mM each of amino acids except arginine, 0.01 mM arginine, 16 μg of mRNA, 0.1 nmol of aminoacyl tRNA, and 2 μL of *E. coli* S30 extract. The mixture was incubated at 37 °C for 60 min.

Each of 1 μL of the reaction mixture was mixed with 19 μL of 50 mM Tris-HCl (pH 6.8), 4% SDS, 2% 2-mercaptoethanol, 12% glycerol, and 0.01% bromophenol blue. The resulting solution was incubated at 95 °C for 5 min, then 5 μL of the solution was applied to a 15% SDS-polyacrylamide gel electrophoresis. After electroblotting to a PVDF membrane, the membrane was incubated at 37 °C for 30 min with 1% BSA in TBST (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, and 0.1% Tween20), then with 1/10 000 diluted T7-Tag Antibody in TBST. After washing with TBST for 5 min three times, the membrane was incubated with 1/5000 diluted alkaliphosphatase-labeled anti-mouse IgG in TBST. The membrane was washed three times with TBST for 5 min, once with TBS (20 mM Tris-HCl (pH 7.5) and 150 mM NaCl), then soaked in NBT/BCIP solution at 37 °C for 30 min.

For quantitative evaluation of the incorporation efficiency, the reaction mixture of wild-type streptavidin was serially diluted with the translation mixture without mRNA, and the resulting 12 samples were applied to the SDS-PAGE (16 × 16 cm, 22 lanes) together with the mutant streptavidins. The band intensity of the Western blot was evaluated by using the public domain NIH Image program (developed at the U. S. National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image/) on Macintosh. Incorporation efficiency of each nonnatural amino acid was expressed as a value obtained from the calibration curve. The eight separete Western blot analyses were made, and the efficiencies were determined by at least four assays.

In a rabbit reticulocyte system, the reaction mixture contained 7 μL of nuclease-treated rabbit reticulocyte lysate, 1.6 μg of mRNA, and 0.1 mM each of amino acids except arginine in 10 μL. The mixture was incubated at 30 °C for 60 min. A 10-μL volume of the reaction mixture was mixed with 10 μL of 100 mM Tris-HCl (pH 6.8), 8% SDS, 4% 2-mercaptoethanol, 24% glycerol, and 0.02% bromophenol blue, then 4 μL of the solution was applied to a 15% SDS-PAGE, followed by the Western blotting.

**Biotin Binding Assay.** The binding activity of the mutant streptavidin was evaluated by a dot blot analysis using biotinylated alkaliphosphatase. The concentrations of the mutant streptavidins in the *E. coli* in vitro translation mixture were obtained from the incorporation efficiencies determined by the Western blot, and 5 ng of each streptavidin was spotted onto a nitrocellulose membrane by using a microfiltration blotting apparatus. After washing with TBS twice for 5 min, the membrane was incubated at 37 °C with 3% gelatin/TBS for 30 min and then with 1/500 diluted biotinylated alkaliphosphatase in 1% gelatin/TBS for 30 min. After washing for 5 min with TBST three times and once with TBS, the membrane was soaked in NBT/BCIP solution at 37 °C for 30 min, then washed with water and dried.

Fluorescence polarization was measured on a BEACON 2000 system (PanVera Corp., Madison, WI) equipped with the filters of 490 nm for excitation and 520 nm for emission. One microliter portion of 1/10 diluted wild-type streptavidin in the *E. coli* in vitro translation mixture was directly added into 100 μL of 0.5 nM fluorescein biotin in TBS. The solution was incubated for 5 min at 25 °C before each measurement.

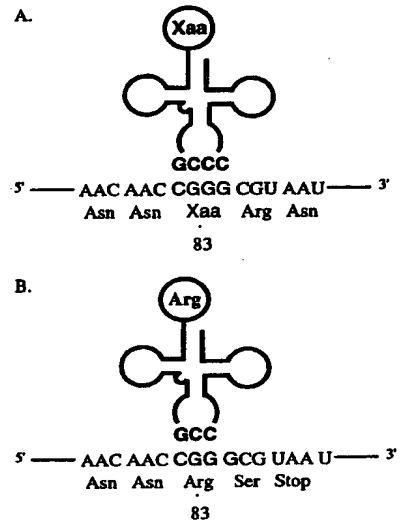


**Figure 2.** Nucleotide and amino acid sequence of the mutated region of streptavidin. (A) In the presence of aminoacyl tRNA$_{CCCG}$, the CGGG four-base codon is read by the tRNA. (B) In the absence of the tRNA$_{CCCG}$, the codon is read as the CGG three-base codon and the protein synthesis stops at the UAA stop codon.

## Results and Discussion

**Expression of Mutant Streptavidins.** The synthetic streptavidin gene was inserted to T7 tag sequence under the control of T7 promoter. The N-terminal T7 tag is introduced for efficient translation in the *E. coli* system and for easy detection by anti T7 tag monoclonal antibody. At the C-terminal, histidine hexamer was attached for the purification of the protein. Since only the full-length protein that carries the terminal histidine hexamer binds to the Ni−NTA column, the truncated peptide that failed to incorporate the nonnatural amino acid can be removed by this procedure.

The mutation was introduced at the Tyr83 site. The resulting sequence is shown in Figure 2. In the presence of the frameshift suppressor tRNA$_{CCCG}$, CGGG will be recognized as a four-base codon and translated to the nonnatural amino acid, then the downstream sequence is translated correctly. On the other hand, in the absence of the tRNA$_{CCCG}$, CGGG is recognized as a three-base codon by Arg-tRNA$_{CCG}$ and the next GCG is translated to Ala, then a UAA stop codon appears. As the result, only and every full-length protein contains the nonnatural amino acid, provided that the tRNA$_{CCCG}$ is not aminoacylated enzymatically with other amino acids during the in vitro protein biosynthesis. One of the advantages of the frameshift strategy is that serious competition with the releasing factor that is encountered in the amber suppression strategy can be avoided.

The coding region including T7 promoter was amplified by a PCR reaction, and the mRNA was prepared by T7 RNA polymerase. The product was analyzed by a denaturing poly-

(8) (a) Hecht, S. M.; Alford, B. L.; Kuroda, Y.; Kitano, S. *J. Biol. Chem.* **1978**, *253*, 4517−4520. (b) Heckler, T. G.; Zama, Y.; Naka, T.; Hecht, S. M. *J. Biol. Chem.* **1983**, *258*, 4492−4495. (c) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Biochemistry* **1984**, *23*, 1468−1473. (d) Heckler, T. G.; Chang, L.-H.; Zama, Y.; Naka, T.; Chorghade, M. S.; Hecht, S. M. *Tetrahedron* **1984**, *40*, 87−94. (e) Lodder, M.; Golovine, S.; Hecht, S. M. *J. Org. Chem.* **1997**, *62*, 778−779.

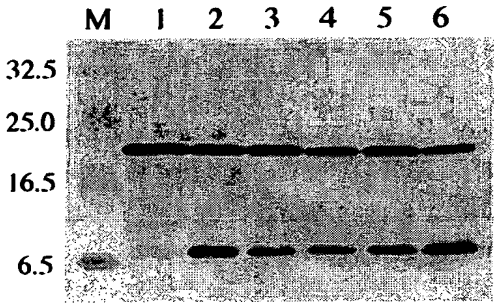


**Figure 3.** Western blot of the reaction mixture of the *E. coli* in vitro translation. The band at 19 kDa is a streptavidin monomer: lane 1, wild-type mRNA; lane 2, mRNA containing CGGU + nitrophenylalanyl tRNA$_{ACCG}$; lane 3, mRNA containing CGGG + nitrophenylalanyl tRNA$_{CCCG}$; lane 4, mRNA containing CGGC + nitrophenylalanyl tRNA$_{GCCG}$; lane 5, mRNA containing CGGA + nitrophenylalanyl tRNA$_{UCCG}$; lane 6, mRNA containing AGGU + nitrophenylalanyl tRNA$_{ACCU}$. Lane M contained prestained molecular weight marker.

acrylamide gel electrophoresis, which showed two bands corresponding to a mRNA terminated at the T7 terminator and that terminated at the 3′ end of the template. The latter will contain the same sequence as the former except for the extra 78 bases after the T7 terminator.

The preparation of aminoacyl tRNA was accomplished by the chemical misacylation method originally developed by Hecht and co-workers.[8] At first, the template of the tRNA$_{ACCU}$(-CA) under T7 promoter was synthesized from two oligonucleotides and then cloned into pUC18. The template of tRNA$_{NCCG}$(-CA) was generated from that of tRNA$_{ACCU}$(-CA) by site-directed mutagenesis. For transcription, the coding region of the plasmid was amplified by the PCR reaction using M13 primer and 3′ terminal primer that defines the 3′ terminal of the tRNA to be lacking a CA dinucleotide. The T7 transcription reaction yielded about 3 mg of the tRNA(-CA) in 1 mL of the reaction mixture after purification with ion-exchange chromatography.

Aminoacyl tRNA was obtained by the coupling of the tRNA-(-CA) with aminoacyl pdCpA catalyzed by T4 RNA ligase. Denaturing polyacrylamide gel electrophoresis showed that about a half of the tRNA(-CA) was linked with the aminoacyl pdCpA irrespective of the type of amino acid. Degradation of aminoacyl pdCpA under the condition of the ligation was followed by HPLC analysis for four typical amino acids, i.e., DL-anthraquinonylalanine, L-ferrocenylalanine, L-2-naphthylalanine, and L-*p*-nitrophenylalanine. The degradation took place, but the rate was not dependent on the type of the amino acids; about 20% after 2 h and 50% after 10 h. These results suggest that the aminoacyl tRNAs charged with different amino acids have been produced by about the same amount and they show similar lifetimes in aqueous media.

Protein synthesis was carried out in *E. coli* S30 in vitro translation system. Amino acids excluding arginine were added at the concentration of 0.1 mM. The concentration of arginine was 0.01 mM. The amount of aminoacyl tRNA was 0.1 nmol per 10 $\mu$L of a reaction mixture for all amino acids examined. The reaction mixture was incubated at 37 °C for 60 min, then the mixture was applied to SDS 15% polyacrylamide gel electrophoresis, followed by the transfer to a PVDF membrane. Streptavidins were detected by using anti T7-tag monoclonal antibody and alkaliphosphatase-labeled anti-Mouse IgG. The amount of wild-type streptavidin was estimated from the measurement of fluorescence polarization. The reaction mixture of the in vitro translation was added to a solution of biotinylated fluorescein ($5 \times 10^{-10}$ M). The amount of streptavidin was determined from a turning point of the fluorescence polarization. It was 10 $\mu$g per 1 mL of the reaction mixture.

Figure 3 shows the results of the Western blotting of the frameshift suppression of AGGU and CGGN four-base codons inserted at the Tyr83 site by the *p*-nitrophenyl tRNA$_{ACCU}$ and tRNA$_{NCCG}$, respectively. The slower migrating band at 19 kDa corresponds to a full-length streptavidin, and the faster migrating band corresponds to a truncated peptide of 9 kDa that is formed when the three of four nucleotide is read by the internal Arg-tRNA. The frameshift suppression of the CGGN codons gave higher yields of the mutant streptavidin than the suppression of the AGGU codon. Since, the CGGG codon gave the highest yield in the CGGN codons, the following experiments were carried out by using the CGGG four-base codon.

**Incorporation Efficiencies of Various Nonnatural Amino Acids in the *E. coli* In Vitro System.** Figure 4 shows results of the Western blotting of the expressions of the mutant streptavidins in the presence of the aminoacyl tRNAs carrying various nonnatural amino acids. In the absence of the tRNA and in the presence of nonaminoacylated tRNA, a negligible amount of full-length streptavidin was synthesized and truncated peptide of 9 kDa was formed, instead. These results show that the in vitro translation system cannot suppress the frameshift mutation unless an aminoacylated tRNA carrying the four-base anticodon is present and that the tRNA$_{CCCG}$ cannot be recognized by any aminoacyl tRNA synthetases.

The Western blotting indicates that the incorporation efficiency markedly depends on the structure of amino acids. On one hand, some aromatic nonnatural amino acids, including those with large side groups such as 2-anthrylalanine and

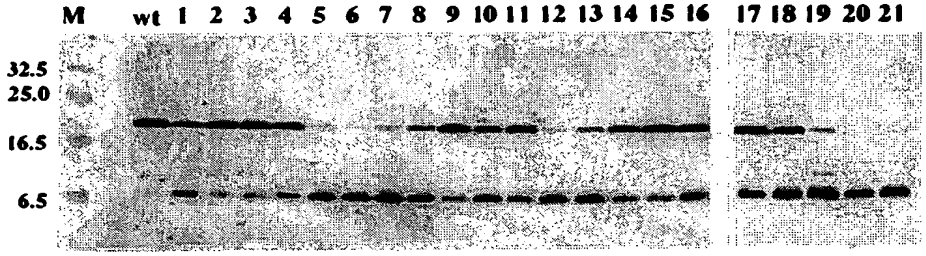


**Figure 4.** Western blot of the reaction mixture of the *E. coli* in vitro translation containing tRNA$_{CCCG}$'s charged with various nonnatural amino acids: lane wt, wild-type mRNA; lane 1, 1-naphthylalanine; lane 2, 2-naphthylalanine; lane 3, *p*-biphenylalanine; lane 4, 2-anthrylalanine; lane 5, 9-anthrylalanine; lane 6, 9-phenanthrylalanine; lane 7, 1-pyrenylalanine; lane 8, 2-pyrenylalanine; lane 9, *p*-nitrophenylalanine; lane 10, 3,5-dinitrophenylalanine; lane 11, *p*-dimethylaminophenylalanine; lane 12, 9-carbazolylalanine; lane 13, 3-(9-ethylcarbazolyl)alanine; lane 14, azatryptophan; lane 15, kynurenine; lane 16, *p*-phenylazophenylalanine; lane 17, *p*-benzoylphenylalanine; lane 18, 2-anthraquinonylalanine; lane 19, ferrocenylalanine; lane 20, no tRNA; lane 21, nonacylated tRNA. Lane M contained prestained molecular weight marker.

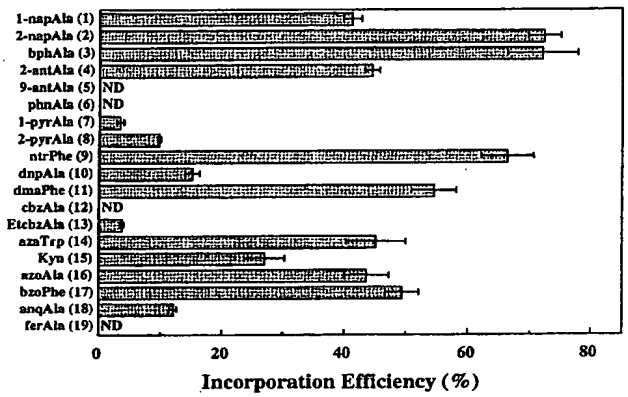


**Figure 5.** Incorporation efficiencies in the *E. coli* in vitro translation by tRNAcccg's charged with various aromatic nonnatural amino acids at Tyr 83 site of streptavidin. The incorporation efficiencies are determined as described in the text. Data are mean ± sem of at least four assays. ND indicates less than 2% efficiency.

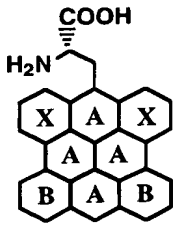


**Figure 6.** Hypothetical model for the adaptability of nonnatural amino acids to the *E. coli* ribosome. The nonnatural amino acids carrying benzene rings in the region A are allowed. Those carrying rings in the region B may also be allowed. Those carrying benzene rings in the region X are rejected.

2-pyrenylalanine, are efficiently incorporated. On the other hand, 9-phenanthrylalanine and ferrocenylalanine are absolutely rejected.

The suppression efficiency was quantitatively evaluated by comparing the band intensities of mutant streptavidins in the Western blotting with the intensities of the serially diluted wild-type streptavidin. Since the detection depends on the binding of the antibody to the N-terminal T7 tag, possible denaturation or partial unfolding of the mutants will not influence the results. The results are summarized in Figure 5.

The most efficient suppression was observed in the case of 2-naphthylalanine (72%). In contrast, 1-naphthylalanine was incorporated in a 30% efficiency. Similarly, 2-anthrylalanine (45%) and 2-pyrenylalanine (10%) were more effectively incorporated than 9-anthrylalanine (less than 2%) and 1-pyrenylalanine (3%), respectively. In the case of carbazole side groups, 3-(9-ethylcarbazolyl)alanine showed a little higher efficiency than 9-carbazolylalanine. These differences suggest that the protein biosynthesizing system discriminates nonnatural amino acids not by their sizes or hydrophobicities but by their shapes of the side groups. It appears that amino acids with linearly expanded aromatic groups such as 2-naphthylalanine, *p*-biphenylalanine, 2-anthrylalanine, *p*-benzoylphenylalanine, and *p*-phenylazophenylalanine are favored. On the other hand, those with rather widely expanded or bend aromatic groups such as 9-anthrylalanine, 9-phenanthrylalanine, and 9-carbazolylalanine are strongly rejected. It should be noted that the efficiencies of 2-anthraquinonylalanine and azatryptophan increase if L-amino acids are used instead of the racemic ones, since only the L-form is expected to be incorporated.

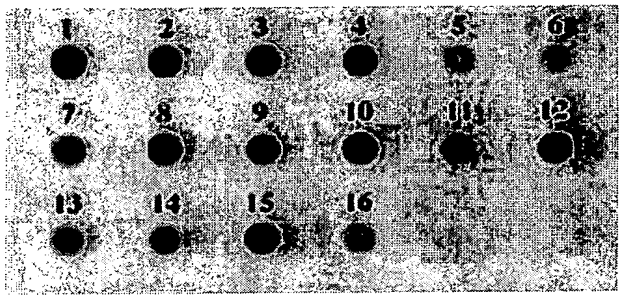


**Figure 7.** Dot blot analysis of wild-type and mutant streptavidins in the reaction mixture of the *E. coli* in vitro translation. A 5 ng sample of each streptavidin was applied. The intensity of each dot reflects the biotin binding activity of the streptavidin. Spot 1 contained the wild-type streptavidin, and spots 2−16 contained mutant streptavidins containing nonnatural amino acids: 2, 1-naphthylalanine; 3, 2-naphthylalanine; 4, *p*-biphenylalanine; 5, 2-anthrylalanine; 6, 1-pyrenylalanine; 7, 2-pyrenylalanine; 8, *p*-nitrophenylalanine; 9, 3,5-dinitrophenylalanine; 10, *p*-dimethylaminophenylalanine; 11, 3-(9-ethylcarbazolyl)-alanine; 12, azatryptophan; 13, kynurenine; 14, *p*-phenylazophenylalanine; 15, *p*-benzoylphenylalanine; 16, 2-anthraquinonylalanine.

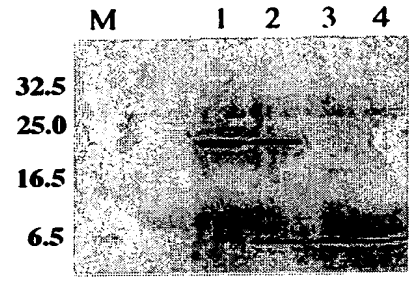


**Figure 8.** Western blot of the reaction mixture of the rabbit reticulocyte in vitro translation: lane 1, wild-type mRNA; lane 2, in the presence of nitrophenylalanyl tRNAcccg; lane 3, in the presence of nonacylated tRNAcccg; lane 4, in the absence of tRNA. Lane M contained prestained molecular weight marker.

Since the amounts and the lifetimes of the aminoacyl tRNAs are about the same for all amino acids, the sharp amino acid dependence of the incorporation efficiency may be governed by the adaptability of the *E. coli* ribosome to each type of nonnatural amino acid. We have reported previously the inhibitory effects of puromycin analogues carrying various nonnatural amino acids instead of *O*-methyl tyrosine.[5] The efficiency of the inhibition by the puromycin analogues and the efficiency of the incorporation in Figure 5 show a good correlation for almost all the nonnatural amino acids tested. The parallel relationship suggests that a selection of the nonnatural amino acids is occurring at the ribosomal A-site.

A close inspection of the amino acid selectivity in Figure 5 suggests a hypothesis for the allowed and excluded regions of the aromatic groups of L-arylalanine-type amino acids by the *E. coli* ribosome (Figure 6). Since *p*-biphenylalanine and 2-naphthylalanine are almost freely accepted by the ribosome, the regions indicated as A must be allowed by the ribosome. Indeed, 2-pyrenylalanine is incorporated moderately despite the large side group. One of the benzene rings of 1-naphthylalanine indicated as X is less favored by the ribosome. A similar tendency is observed in the comparison of 1- and 2-pyrenylalanine. The rejection of 9-phenanthrylalanine is also explained in terms of the excluded benzene ring X. The benzene ring indicated by B may belong to an allowed region because it is allowed both in 2-anthrylalanine and in *p*-benzoylphenylalanine.

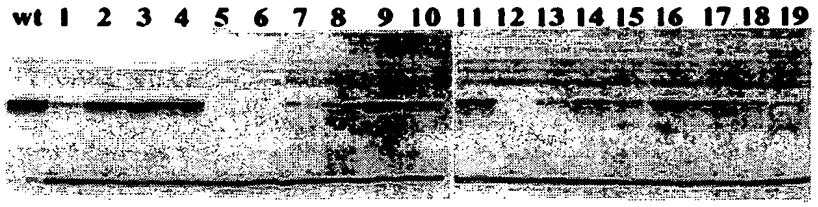


**Figure 9.** Western blot of the reaction mixture of the rabbit reticulocyte in vitro translation containing aminoacyl tRNAcccg's carrying various nonnatural amino acids: lane wt, wild-type mRNA; lane 1, 1-naphthylalanine; lane 2, 2-naphthylalanine; lane 3, *p*-biphenylalanine; lane 4, 2-anthrylalanine; lane 5, 9-anthrylalanine; lane 6, 9-phenanthrylalanine; lane 7, 1-pyrenylalanine; lane 8, 2-pyrenylalanine; lane 9, *p*-nitrophenylalanine; lane 10, 3,5-dinitrophenylalanine; lane 11, *p*-dimethylaminophenylalanine; lane 12, 9-carbazolylalanine; lane 13, 3-(9-ethylcarbazolyl)alanine; lane 14, azatryptophan; lane 15, kynurenine; lane 16, *p*-phenylazophenylalanine; lane 17, *p*-benzoylphenylalanine; lane 18, 2-anthraquinonylalanine; lane 19, ferrocenylalanine.

The information on the allowed and excluded rings will serve for designing new nonnatural amino acids that can be incorporated in the in vitro system.

**Activities of Mutant Streptavidins Carrying Various Nonnatural Amino Acids at the Tyr83 Site.** The biotin binding activities of the mutant streptavidins were evaluated by dot blot analysis using biotinylated alkaliphosphatase. The concentrations of the mutant streptavidins in the in vitro translation mixture were obtained from the efficiencies in Figure 5, and 5 ng of each streptavidin was applied to the dot blot analysis. As shown in Figure 7, the incorporation of small amino acids such as naphthylalanines and *p*-nitrophenylalanine did not affect the binding activity very much. However, the incorporation of large aromatic groups such as anthryl and pyrenyl groups reduced the binding activity. Although the Tyr83 site is far from the biotin binding site, the introduction of large amino acids would influence the structure of the binding site and reduce the binding activity.

**Incorporation Efficiencies of Various Nonnatural Amino Acids in the Rabbit Reticulocyte Lysate System.** Incorporation efficiencies were also examined in rabbit reticulocyte lysate to explore the toleration in the eukariote protein biosynthetic system. As shown in Figure 8, the full-length streptavidin was synthesized only in the presence of tRNAcccg aminoacylated with nitrophenylalanine, indicating that the nonnatural amino acid was incorporated into the protein under direction of the four-base codon in the rabbit reticulocyte system as well as in the *E. coli* system. The Western blotting in Figure 9 shows that various aromatic nonnatural amino acids are incorporated into streptavidin in the reticulocyte lysate system. It must be noted that 9-anthrylalanine, 9-phenanthrylalanine, 9-carbazolylalanine, and ferrocenylalanine could be incorporated neither in the rabbit system nor in the *E. coli* system.

The efficiencies quantitatively evaluated from the Western blotting were summarized in Figure 10. The comparisons between 1- and 2-naphthylalanine, 2- and 9-anthrylalanine, 1- and 2-pyrenylalanine, and 3-(9-ethylcarbazolyl)alanine and 9-carbazolylalanine suggest that nonnatural amino acids with linearly expanded aromatic groups are more favorable than those with widely expanded or bend aromatic groups as has been observed in the *E. coli* system. The parallel amino acid dependence in the two in vitro systems suggests that the ribosome of rabbit reticulocyte recognizes the nonnatural amino acids in the same manner as that of *E. coli*. Presumably, the structures of the A-sites of the two types of ribosomes are well preserved.

A detailed comparison of the incorporation efficiencies in the two systems, however, discloses a subtle but important difference. The ratios of the incorporation efficiencies in the rabbit system to those in the *E. coli* are shown in Figure 11. It

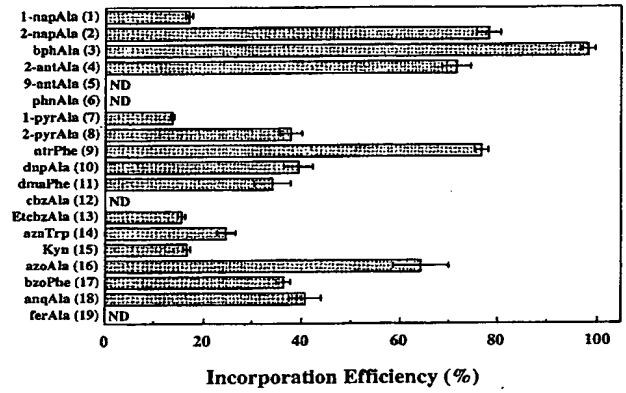


**Figure 10.** Incorporation efficiencies in rabbit reticulocyte in vitro translation by tRNAcccg's charged with various aromatic nonnatural amino acids at the Tyr 83 site of streptavidin. ND indicates less than 10% efficiency.

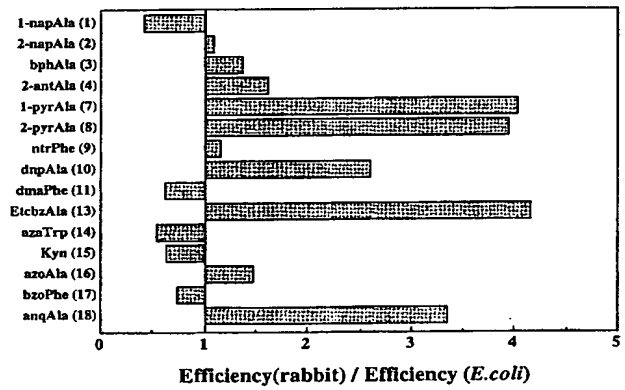


**Figure 11.** Ratios of the incorporation efficiencies of nonnatural amino acids in rabbit reticulocyte system to those in the *E. coli* system.

is seen that the amino acids with large side groups such as pyrene, dinitrobenzene, and anthraquinone are incorporated in the rabbit system more efficiently than in the *E. coli* system. This suggests that the molecular recognition of the rabbit ribosomal A-site is less tight than that of *E. coli* and the former system is more appropriate for preparing proteins incorporated with nonnatural amino acids carrying relatively large side groups.

The efficient incorporation of the aromatic nonnatural amino acids described in this paper will open a way to investigate fluorescence analyses, photoinduced electron transfers, photoenergy transfers, and other specialty functions on the protein framework.

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**Supporting Information Available:** Experimental details of the syntheses of nonnatural amino acids, aminoacyl pdCpAs, tRNA(-CA)s, and mRNAs and HPLC profiles of *p*-nitrophenylalanyl pdCpA (13 pages, print/PDF). See any current masthead page for ordering information and Web access instructions.

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# Cell-free N-terminal protein labeling using initiator suppressor tRNA

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**Abstract**

A highly efficient method for the introduction of fluorophores and other markers at the N terminus of proteins produced in a cell-free extract has been developed. The method utilizes an amber (CUA) initiator suppressor tRNA chemically aminoacylated with a fluorophore–amino acid conjugate which is introduced into an *Escherichia coli* S30 cell-free translation system. The DNA template contains a complementary amber (UAG) codon instead of the normal initiation (AUG) codon. Using this approach, the fluorophore BODIPY-F1 (4,4-difluoro-5,7-dimethyl-4-bora-3a,4a- diaza-*s*-indacene-3-propionic acid) has been incorporated at the N terminus of several model proteins. The specific labeling achieved (27–67%) using this approach is much higher than that of wild-type tRNAs. Several potential biophysical and biotechnological applications of this new technology are described.


*Keywords:* Fluorophore; Protein labeling; Cell-free expression; Initiator suppressor tRNA; BODIPY-FL

Protein engineering is widely used in basic research and biotechnology to obtain proteins with improved or new properties. In addition to conventional mutagenesis other techniques, such as chemical or enzymatic modification, protein evolution [1] or incorporation of non-native amino acids in cellular [2,3] or cell-free [4,5] expression systems, are used to generate novel proteins. An approach based on site-specific nonnative amino acid replacement provides the ability to incorporate nonnative amino acids into proteins during cell-free expression [4]. Furthermore, since nonnative amino acids can be designed as markers and affinity tags, this approach can be used to produce proteins for diverse biophysical and biotechnological applications [6]. For example, fluorophore-labeled proteins are suitable for detection with laser-induced fluorescence in conjunction with gel scanners [7] or capillary electrophoresis [8]. Affinity markers incorporated into proteins, such as biotin, or photocleavable biotin [9,10] could be used for purification and oriented, reversible attachment to surfaces.

The α-amino group of the N-terminal amino acid is an ideal site for the marker moiety, because this group is usually not essential for protein function [11,12]. Recently, we have described the incorporation of the fluorophore, BODIPY-FL-Met at the N terminus of nascent proteins using in vitro translation in an *Escherichia coli* S30 coupled transcription–translation system and misaminoacylated wild-type initiator tRNA [13]. Although this approach is suitable for fluorescent detection of proteins in gels, the specific labeling achieved is low (1–2%) due to the competition between the exogenenously added BODIPY-FL-Met-tRNA$^{fmet}$ and the endogenous fMet-tRNA$^{fmet}$.

To avoid this competition, we have now designed and evaluated a system utilizing an mRNA template with an amber codon (UAG) instead of a normal initiating codon (AUG) and a complementary misaminoacylated initiator suppressor tRNA capable of initiating protein synthesis from the UAG codon. A basis for this approach is the work of Varshney and RajBhandary [14]. These studies demonstrated that initiator tRNAs with

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altered anticodons are capable of initiating protein synthesis with amino acids other than methionine. For example, an initiator tRNA with a "CUA" (amber) anticodon ($tRNA^{fMet2}(CUA)$) is capable of initiating protein translation from a "UAG" amber codon on an mRNA template in both in vivo and in vitro expression systems [14–16].

Since the *E. coli* mutant initiator tRNA is a poor substrate for aminoacyl synthetase [14], we used the chemical aminoacylation approach for the preparation of mutant initiator tRNA aminoacylated with fluorophore–amino acid conjugate. The chemical aminoacylation method has been previously used for the preparation of a variety of elongator suppressor tRNAs aminoacylated with nonnative amino acids [5,17–19]. Here we present a cell-free expression labeling system that utilizes amber suppressor $tRNA^{fMet2}(CUA)$ (fma-tRNA) misaminoacylated with BODIPY-FL-Met or BODIPY-FL-Val and mutant genes containing a UAG stop codon ("amber at position 1 or "amber-1") instead of a normal initiation codon (AUG).

## Materials and methods

*E. coli* T7 S30 extract translation system, restriction endonucleases *Kpn*I and *Hin*dIII, and pGEM-3Z vector DNA were from Promega (Madison, WI). T4 DNA ligase, T4 RNA ligase, and restriction endonuclease *Dpn*I were from New England Biolabs (Beverly, MA), *Pfu* turbo DNA polymerase and XL-Blue-1 competent cells were from Stratagene (LaJolla, CA). pETblue-2 vector was from Novagen (Madison, WI). Oligonucleotides were from Sigma–Genosys (The Woodlands, TX), QIAEX II gel extraction kit, *Taq* PCR master mix kit, and QIAquick PCR purification kit were from Qiagen (Valencia, CA). TALON-$Co^{2+}$ metal affinity resin was from Clontech (Palo Alto, CA). Snake venom phosphodiesterase I (SVPDE I)[1] was from Worthington Biochemical Corp. (Freehold, NJ).

### *Cloning of E. coli initiator tRNA gene*

A 420-bp fragment of nusA *E. coli* gene containing the gene for $tRNA_2^{fMet}$ [20] was amplified using primers CACTACTGCAAGATTTTACGTCCGTCTCGG and CCACAAAGCTTCAAAACCCAGGGCCTCAAC from *E. coli* genomic DNA introducing *Pst*I and *Hin*dIII sites. Obtained fragment was purified using QIAquick kit, digested by *Pst*I/*Hin*dIII, and cloned into *Pst*I/*Hin*dIII site of pGEM-3 vector to give pGEM-fmw. Amber suppressor mutant $tRNA_2^{fMet}$ gene ($A_{35} \rightarrow U$; $U_{36} \rightarrow A$) was prepared using QuickChange kit (Stratagene) and primers 5′-GGTAGCTCGTCGGGCTCTA AACCCGAAGATCGTCGG-3′ and 5′-CCGACGAT CTTCGGGTTTAGAGCCCGACGAGCTACC-3′ to give pGEM-fma. The sequence of inserts for both pGEM-fmw and pGEM-fma was confirmed by dideoxy sequencing.

### *fma-tRNA isolation*

Amber suppressor $tRNA_2^{fMet}$ (fma-tRNA) was isolated from *E. coli* cells transformed by pGEM-fma according to described procedure [15] using phenol extraction followed by nondenaturing polyacrylamide gel electrophoresis and "crush and soak" elution with 50 mM sodium acetate, pH 5.0, 1% SDS, 2 mM EDTA, and ethanol precipitation.

### *Preparation of fma-tRNA(-CA)*

Ten $OD_{260}$ of fma-tRNA was dissolved in 500 µl of 100 mM Tris–HCl buffer, 100 mM NaCl, 15 mM $MgCl_2$, pH 8.9. The solution was pre-chilled to 0 °C and then 5 U of SVPDE I was added. The mixture was incubated for 30 min at 0 °C and then extracted by vortexing with 500 µl of phenol saturated with 1 mM NaOAc, pH 5.0. The phases were separated by centrifugation, the aqueous layer was transferred to a new tube, and the tRNA was precipitated by the addition of 1000 µl of ethanol followed by incubation at −20 °C for 30 min. The pellet was isolated by centrifugation and dissolved in 50 µl of water.

### *Preparation of BODIPY-FL-Met-fma-tRNA and BODIPY-FL-Val-fma-tRNA*

Ligations were carried out using 200 µl of T4 RNA ligase buffer (New England Biolabs), 10–30% DMSO, 10 $OD_{260}$ of fma-tRNA(-CA), 2 $OD_{260}$ of BODIPY-FL-Met-pdCpA or BODIPY-FL-Val-pdCpA [21], and 400 U of T4 RNA ligase. Reactions were incubated for 18 h at 4 °C and tRNAs were precipitated by addition of 0.1 vol of 3 M NaOAc (pH 4.5) and 3 vol of ethanol. The pellet was isolated by centrifugation, dissolved in 100 µl of water, and analyzed and purified using RP-HPLC as described earlier [13]. Pooled fractions containing ligated tRNA were concentrated to <0.5 ml and desalted on NAP-5 gel filtration column (Amersham Biosciences, Piscataway, NJ). BODIPY-FL-Met-$tRNA^{fMet}$ and BODIPY-FL-Lys-$tRNA^{Lys}$ were prepared as previously described [13,22].

### *Construction of the plasmids for in vitro expression*

pT7-WT-6xHis-αHL plasmid DNA coding for α-hemolysin (αHL) [23] was kindly supplied by Prof.

[1] *Abbreviations used:* SVPDE I, snake venom phosphodiesterase I; DMSO, dimethyl sulfoxide; αHL, α-hemolysin; DHFR, dihydrofolate reductase; GST, glutathione *S*-transferase; DTT, dithiothreitol; WT, wild-type.

Hagan Bayley (Texas A&M University). Plasmid pQE-16 coding for the mouse dihydrofolate reductase (DHFR) gene was purchased from Qiagen, pBEST*luc* plasmid for firefly luciferase expression was purchased from Promega Corp. The genes coding for human glutathione *S*-transferase (GST) and calmodulin were amplified from human genomic DNA and inserted into the pETblue-2 vector. The QuickChange kit (Stratagene) for site-directed mutagenesis was used for substitution of ATG initiation codons for TAG amber codons. The presence of mutations was verified by DNA sequencing. Plasmid DNAs for in vitro translations were isolated using Qiagen plasmid isolation kit.

*Cell-free protein synthesis and detection*

In a typical experiment in vitro translations were carried out in a 10-μl reaction mixture containing 3 μl of *E. coli* T7 S30 extract, 4 μl of premix, 1 μl of 1 mM complete amino acids mixture, 0.5–1 μg of plasmid DNA, and 0.2–2 μg of fluorescently labeled tRNAs. The reaction mixtures were incubated at 37 °C for 1 h. Aliquots of 1–2 μl were diluted to 25 μl with 1× SDS-gel loading buffer (62 mM Tris–HCl, 1% SDS, 100 mM DTT, 0.01% bromophenol blue, 10% v/v glycerol, pH 6.8) and subjected to gradient 8–16% SDS–PAGE using precast gels (ISC BioExpress, Kaysville, UT). After electrophoresis the gels were scanned using a FluorImager SI (Molecular Dynamics) equipped with an argon laser (488 nm). Bands were quantitated using ImageQuant software (Molecular Dynamics).

*Purification of 6xHis tagged proteins*

The translations of amber-1 6xHis-α-HL, 6xHis-GST, and 6xHis-calmodulin were carried out at 500-μl scale (37 °C, 1 h). The translation mixture was mixed with 200 μl of Talon–Sepharose beads equilibrated with 50 mM Hepes–Na, pH 7.6. After 30 min of incubation at room temperature the mixture was loaded onto a 2-ml disposable column (Clontech) and washed with 4× 500 μl of the 50 mM Hepes–Na, 5 mM imidazole (pH 7.6) buffer. The 6xHis tagged proteins were eluted with elution buffer (50 mM Hepes–Na, 300 mM imidazole, pH 7.6). Fractions of 100 μl were collected and 5-μl aliquots were analyzed by SDS–PAGE/FluorImager. Fractions containing highest amount of fluorescence were pooled (300 μl volume) and dialyzed against 500 ml of 50 mM Hepes–Na, pH 7.6, using microdialyzers (3500 MWCO, Pierce Chemical Co., Rockford, IL).

The translation of amber-1 6xHis-DHFR was carried out at 500-μl scale (37 °C, 1 h). After the translation was complete, a solution of 500 μl of 8 M urea was added followed by 200 μl of Talon–Sepharose beads equilibrated with 4 M urea, 200 mM Tris–HCl, 500 mM NaCl (pH 8.0) and the resulting mixture was incubated at room temperature for 30 min. The suspension was then transferred to a 2 ml disposable column (Clontech) and washed with 4× 500 μl of 4 M urea, 500 mM NaCl, 200 mM Tris–HCl, pH 8.0. DHFR was eluted by the same buffer containing in addition 200 mM imidazole. Fractions of 100 μl were collected and 5-μl aliquots were analyzed by SDS–PAGE/FluorImager. Fractions containing highest amount of fluorescence were pooled (300 μl volume) and dialyzed against 500 ml of 4 M urea, 50 mM Hepes–Na, pH 7.6.

*Protein quantitation and determination of specific labeling*

For the determination of specific labeling, three model proteins (trypsin inhibitor, chicken egg albumin, carbonic anhydrase; Sigma, Milwaukee, WI) were chemically labeled with BODIPY-FL, SE (Molecular Probes, Portland, OR) and purified by gel filtration using NAP-5 columns. The concentration of proteins and fluorophore/protein ratio was determined by measuring UV–Vis absorption at 280 and 505 nm. Various amounts of BODIPY-FL-labeled proteins were then analyzed by SDS–PAGE and FluorImager (488-nm excitation). The signal intensity was plotted against the amount of BODIPY-FL fluorophore loaded on the gel and used as a calibration curve for the quantitation of the in vitro-produced BODIPY-FL-labeled proteins. For the determination of total amount of in vitro-produced proteins, model proteins were diluted serially (120–7.5 ng/band), separated on 8–16% SDS–PAGE, and stained using SYPRO Orange (Molecular Probes) according to the manufacturer; band intensities were measured using FluorImager (488-nm excitation). The calibration curve was then used for the determination of the total amount of protein produced in an in vitro system. The presence of the BODIPY-FL label on the protein does not interfere with the protein quantitation using the SYPRO Orange stain (data not shown). The read-through determination was performed by assembling translation mixtures in total volume of 10 μl containing 0.5 μg of DNA template and 0.25 μl (0.18 μg) of BODIPY-FL-Lys-tRNA$^{Lys}$. Translation was performed in the absence or presence of 1 μg of purified, uncharged fma-tRNA. The translation mixtures were then separated on SDS–PAGE and imaged, and protein bands were quantitated using the FluorImager. The intensities were normalized to 100% (the intensity of the full-length protein band using WT template and BODIPY-FL-Lys-tRNA$^{Lys}$).

## Results

*Preparation and characterization of misaminoacylated tRNAs*

Previously RajBhandary and co-workers [15] reported a 15- to 20-fold overexpression of fma-tRNA

using an M13 recombinant phage expression system. Purification to near homogeneity was achieved using preparative PAGE. For fma-tRNA expression we used a high-copy-number pGEM-3 vector that provided a similar level of overproduction as judged by nondenaturing PAGE (data not shown). The fma-tRNA was isolated using previously described procedures [15] and appeared as a single band on 8% denaturing PAGE after ethidium bromide staining. On average, the yield of purified fma-tRNA was about 40 $OD_{260}$ per 1 liter of *E. coli* culture.

To selectively remove the last two nucleotides (CA) from the 3′ end of fma-tRNA we used snake venom phosphodiesterase I from *Crotalus adamanteus*. This enzyme successively hydrolyzes 5′ mononucleotides from 3′-hydroxy-terminated ribo- and deoxyribooligonucleotides [24]. SVPDE I has been shown to produce defined-length digests from tRNA at low temperature and with limiting amounts of enzyme [25,26]. This method was used previously for the preparation of the tRNAs lacking two nucleotides at the 5′ terminus for the purpose of "chemical aminoacylation" [27]. To optimize fma-tRNA truncation, the digestion by SVPDE I was monitored at various time intervals (0–40 min) on a denaturing 8% PAGE followed by ethidium bromide staining (Fig. 1). This experiment demonstrates that nearly quantitative removal of the first two nucleotides is achieved after 30 min of incubation and no nonspecific degradation products are observed. These conditions were then used for fma-tRNA(-CA) preparation at a scale up to 20 $OD_{260}$.

In the next step, fma-tRNA(-CA) was ligated to BODIPY-FL-Met(Val)-pdCpA and the reaction mixtures were analyzed by HPLC [13]. Under standard conditions (1× ligation buffer as supplied by manufacturer, 10% DMSO, 4 °C, 12–18 h) product yield for BODIPY-FL-Val-pdCpA was 70–80% of the starting fma-tRNA(-CA). In contrast, under the same conditions the yield of product with BODIPY-FL-Met-pdCpA was only 30–40%. After increasing the concentration of DMSO in the ligation mixture to 30%, the ligation yields for both Met and Val conjugates were in the 70–80% range. Ligated tRNAs were purified by preparative HPLC to homogeneity, concentrated, desalted, and used in translation experiments. The purified BODIPY-FL-Met-fma-tRNA and BODIPY-FL-Val-fma-tRNA were further characterized by their $A_{260}/A_{505}$ absorption ratio (~10), which is very close to the theoretical value for a 1:1 BODIPY-FL/fma-tRNA conjugate.

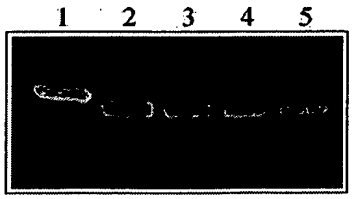


Fig. 1. Time course of fma-tRNA digestion by SVPDE I analyzed by denaturing PAGE and imaged by ethidium bromide staining/fluorescence; lane 1, starting material; lanes 2–5, digestion for 5, 10, 20, and 40 min, respectively.

### *BODIPY-FL-Met incorporation into α-HL using wild-type and amber-1 template*

BODIPY-FL-Met-fma tRNA was evaluated in a T7 S30 transcription/translation system (see Materials and methods) for its ability to incorporate fluorophore into cell-free produced proteins using various DNA templates. The *E. coli* BODIPY-FL-Met-$tRNA^{fMet}$ [13] and BODIPY-FL-Lys-$tRNA^{Lys}$ [22] were also used for comparison purposes (see Materials and methods). For templates, two α-HL DNA constructs (WT and amber-1) were used (see also Materials and methods). The amber-1 template construct contains an "amber" (UAG) codon instead of a normal initiation codon, whereas the WT template contains a normal AUG start initiator codon at the same position. In the "amber-1" case, the protein synthesis is expected to be initiated only when the complementary aminoacylated "amber" initiator tRNA is present in the translation system. Translation reactions in T7 S30 transcription/translation system containing various combinations of DNA templates and fluorescent tRNAs were performed and after 30-min incubation analyzed using SDS–PAGE and fluorescent scanning. The results of these experiments are shown in Fig. 2. As seen, a band with an apparent MW of 30 kDa, corresponding to the full length of α-HL, is observed in lane 4 (WT α-HL/BODIPY-FL-Met-$tRNA^{fMet}$), lane 5 (WT α-HL/BODIPY-FL-Lys-$tRNA^{Lys}$), and lane 9 (amber-1 α-HL/BODIPY-FL-Met-fma-tRNA). No fluorescent protein products were observed when translation reactions did not contain DNA template (lanes 1–3), which indicates that endogenous templates are not present in the system. As expected, no fluorescent protein products were detected in the case when the reaction contained normal (WT) template and the

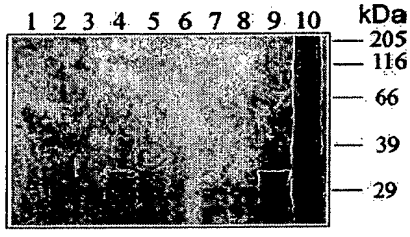


Fig. 2. Translation of α-HL wild-type (lanes 4–6) and amber-1 (7–9) DNA templates in the *E. coli* T7 S30 extract, analyzed on SDS–PAGE and imaged by laser scanner. Translations performed in the presence of BODIPY-FL-Met-$tRNA^{fMet}$ (lanes 1, 4, 7), BODIPY-FL-Lys-$tRNA^{Lys}$ (lanes 2, 5, 8), and BODIPY-FL-Met-fma-tRNA (lanes 3, 6, 9); lanes 1–3 contain no DNA template.

BODIPY-FL-Met-fma-tRNA (lane 6). The highest fluorescent intensity for the α-HL band was observed in the case of the amber-1 α-HL template and BODIPY-FL-Met-fma-tRNA combination (lane 9). Weak fluorescent bands with an apparent MW of 26 kDa in the cases of amber-1 DNA template and BODIPY-FL-Met-$tRNA^{fMet}$ (lane 7) and BODIPY-FL-Lys-$tRNA^{Lys}$ (lane 8) correspond to β-lactamase. These results indicate that the N-terminal labeling can be accomplished by utilizing an "amber-1 labeling system" (i.e., amber-1 α-HL template and BODIPY-FL-Met-fma-tRNA). Furthermore, since the highest fluorescent intensity was observed for the amber-1 system (Fig. 2, lane 9) this approach appears to be an efficient method for labeling proteins.

*Comparison of BODIPY-FL-Met- and BODIPY-FL-Val incorporation at the N-terminus of α-HL*

A set of experiments was performed to compare the ability of the fma-tRNA misaminoacylated with BODIPY-FL-Met or with BODIPY-FL-Val to initiate a translation of amber-1 α-HL. Aliquots from reaction mixtures containing equal amounts of BODIPY-FL-Met-fma-tRNAor BODIPY-FL-Val-fma-tRNA were analyzed by SDS–PAGE and the intensity of fluorescent protein bands was measured using Fluoroimager. As seen in Fig. 3, a 20–30% higher intensity is observed for the case of BODIPY-FL-Val-fma (lanes 5 and 6) compared to that of BODIPY-FL-Met-fma-tRNA (lanes 3 and 4). This result indicates that BODIPY-FL-Val produces a 20–30% higher yield of labeled protein compared to BODIPY-FL-Met. This may be due to several factors, such as increased stability and/or better utilization/incorporation of the BODIPY-FL-Val-fma-tRNA conjugate by the translational machinery.

*General applicability of in vitro amber-1 labeling system*

In addition to α-HL, the ability to incorporate BODIPY-FL fluorophore at the N terminus of several other proteins was investigated. The plasmids coding for wild-type and amber-1 genes of these proteins were translated in an *E. coli* S30 transcription/translation system in the presence of either BODIPY-FL-Lys-$tRNA^{Lys}$ or BODIPY-FL-Val-fma-tRNA. Equal aliquots of the reaction mixtures were then separated on SDS–PAGE and analyzed by fluorescent imaging. As shown in Fig. 4, the amber-1 labeling system (Fig. 4A) results in all cases in fluorescent protein bands of much higher intensity compared to labeling using the wild-type gene and BODIPY-FL-Lys-$tRNA^{Lys}$ (Fig. 4B). The apparent increases in the intensity of these bands are 20-fold for DHFR, 10-fold for α-HL and calmodulin, 8-fold for GST, and 2-fold for luciferase. No other significant fluorescent bands are observed. In addition to an apparent increase in the efficiency of labeling (see below) the amber-1 initiation of luciferase (Fig. 4A, lane 4) eliminates a band normally observed in the in vitro expression of WT luciferase [28] and caused by internal initiation of the protein synthesis.

*Determination of specific labeling and protein yield*

Experiments were performed to determine the extent of specific labeling in the amber-1 expression system and the level of protein expression. For this purpose,

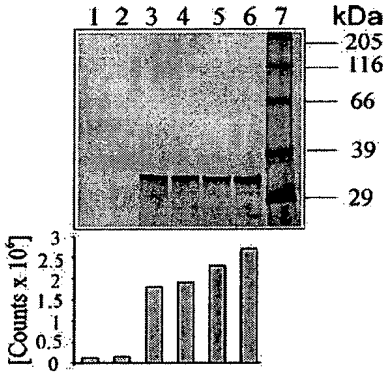


Fig. 3. Translation of α-HL amber-1 template in the *E. coli* T7 S30 extract, separated on SDS–PAGE and imaged by laser scanner. Translations performed in the presence of BODIPY-FL-Met-fma-tRNA (lanes 3 and 4, 0.5 and 1.0 μg, respectively) and BODIPY-FL-Val-fma-tRNA (lanes 5 and 6, 0.5 and 1.0 μg, respectively). Lanes 1 and 2: translation in the absence of DNA template; lane 1, BODIPY-FL-Met-fma-tRNA (0.5 μg); lane 2, BODIPY-FL-Val-fma-tRNA (0.5 μg). Graph illustrates relative intensity of bands on gel as measured using FluorImager.

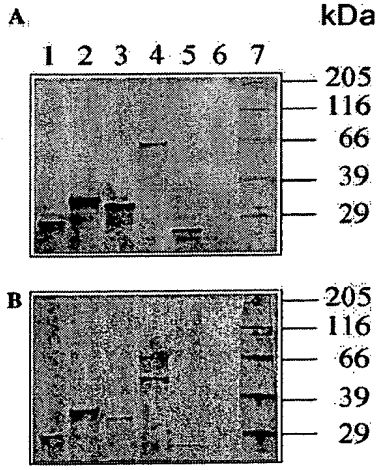


Fig. 4. Translation of amber-1 (A) and WT (B) DNA templates in the presence of BODIPY-FL-Val-fma tRNA (A) and BODIPY-FL-Lys $tRNA^{Lys}$ (B). Lane 1, calmodulin; lane 2, α-hemolysin; lane 3, GST; lane 4, luciferase; lane 5, DHFR; lane 6, no DNA template; lane 7, fluorescent protein markers.

aliquots of several in vitro-produced proteins (α-hemolysin, GST, calmodulin) were purified using a 6xHis tag as described under Materials and methods. Samples were then separated on 8–16% SDS gradient gels and the fluorescent bands were quantitated using FluorImager. The amount of BODIPY-FL-labeled protein was determined in each case using a calibration curve (Fig. 5) obtained from several BODIPY-FL-labeled proteins containing known amounts of fluorophore (see Materials and methods for details). To determine the total amount of protein produced, the gel was then stained after scanning with SYPRO Orange and intensity of the bands quantitated again using FluorImager. The SYPRO Orange assay was calibrated using a mixture of protein standards (see Materials and methods for details). Both measurements combined with the analysis of crude reaction mixture prior to purification allow the determination of specific labeling and protein yield.

The results of these measurements are presented in Table 1. As can be seen the labeling efficiency for the different proteins examined varied from 27 to 67%. The nonquantitative labeling observed is likely due to the recycling of the labeled fma-tRNA by translational machinery and reaminoacylation with nonlabeled amino

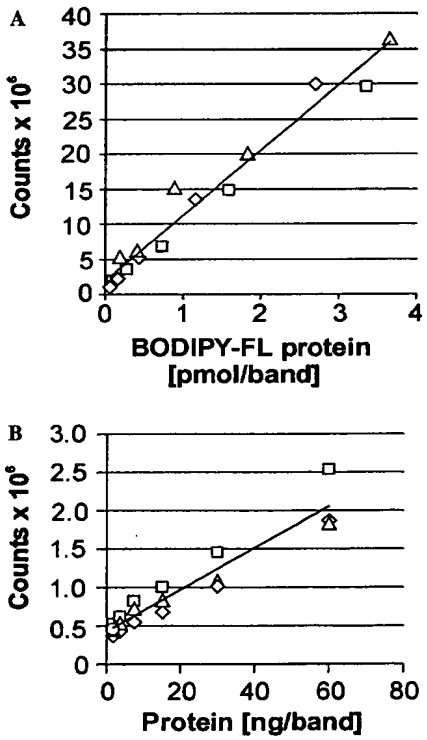


Fig. 5. Calibration curves for model proteins. Three model unlabeled (A) and BODIPY-FL labeled (B) proteins were separated on SDS–PAGE and the intensities of the bands quantified using SYPRO Orange (A) or BODIPY-FL signal (B). Solid lines are calibration curves based on averaged signals of three proteins.

Table 1
Labeling efficiency and protein yield

| Amber-1 protein | Fluorophore incorporation [27] (%) | Protein yield (μg/ml) |
|---|---|---|
| α-Hemolysin | 53 | 10 |
| Calmodulin | 67 | 7 |
| Glutathione *S*-transferase | 27 | 5 |

Translations of various amber-1 DNA templates were performed in the presence of BODIPY-FL-Val-fma tRNA and the resulting protein products were purified using 6xHis tag. The fluorophore incorporation and the total protein yield were determined as described under Materials and methods.

acids. Overall, the yields of the nascent proteins in the amber-1 translation system are approximately five times lower than the yields previously obtained for the in vitro expression of the wild-type templates (data not shown).

To further investigate nonquantitative labeling in the amber-1 suppression system, additional in vitro translations were performed using amber-1 DNA templates and elongator tRNA (BODIPY-Lys-tRNA$^{Lys}$). This experimental design allows detection of possible protein production from the amber-1 DNA template *in the absence* of *complementary suppressor tRNA* (fma-tRNA). Similar translation reactions which contained nonaminoacylated fma-tRNA in addition to labeled elongator tRNA (BODIPY-Lys-tRNA$^{Lys}$) were also performed to assess the capability of the S30 extract to aminoacylate/initiate translation that might lead to N-terminal label dilution. As a control, the translation of WT templates was performed in the presence of the BODIPY-Lys-tRNA$^{Lys}$. The translations were analyzed by SDS–PAGE and fluorescent imaging and the intensity of the full-length protein bands normalized to 100% (WT template).

As seen in Table 2, these experiments reveal that initiation of the protein translation occurs at a significant level ranging between 8 and 21% even *in the absence of the complementary suppressor tRNA* (fma-tRNA). Upon addition of the nonaminoacylated fma-tRNA the level of protein production increases to 38–80%. These results clearly indicate that the observed label dilution in the amber-1 labeling system can be attributed to the presence of tRNAs in the S30 system that are able to

Table 2
Read-through values for amber-1 systems without fma-tRNA and with nonaminoacylated fma-tRNA

| Amber-1 protein | Read-through (−tRNA) (%) | Read-through (+tRNA) (%) |
|---|---|---|
| α-Hemolysin | 8 | 38 |
| Calmodulin | 8 | 72 |
| Glutathione *S*-transferase | 21 | 80 |

initiate protein synthesis from the amber-1 DNA template. A second, even more significant, factor leading to N-terminal label dilution is reaminoacylation of the fma-tRNA due to endogenous aminoacyl synthetases present in the reaction mixture.

## Discussion

This paper describes a system for labeling proteins at their N-terminal end using cell-free expression and amber suppression. This system offers significant advantages over existing methods for cell-free protein labeling. As discussed above, the labeling efficiency ranged from 27 to 67%, which is a significant improvement over existing methods using elongator or wild-type initiator tRNAs. The attachment of a single label at the N terminus is highly desirable for a variety of applications. For example, a biotin attached to the N-terminal position can provide a convenient method for oriented immobilization of in vitro-produced proteins. The oriented immobilization should be useful in the preparation of functional protein microarrays [29]. In the case of fluorescent labeling, the placement of a single fluorophore at the N terminus is also very advantageous. While chemical labeling methods often produce multiple species with different isoelectric points, the amber-1 labeling system should produce single species with isoelectric points very close to those of unmodified species. This can be an important advantage for affinity capillary electrophoresis, where small shifts in mobility due to the binding of a ligand to a protein is to be detected [8].

In an earlier paper [13], an N-terminal labeling system based on the BODIPY-FL-Met-tRNA$^{fMet}$ was reported. However, this system exhibited significantly lower efficiency (%) because native unlabeled Met-tRNA$^{fMet}$ competed with the labeled tRNA for the start codon (AUG), thereby causing label dilution. Hardesty and co-workers [30–32] also utilized aminoacylated wild-type initiator tRNA conjugated to a variety of fluorophores to study its interaction with initiation factor 2, ribosomes, and the interaction of the nascent polypeptide with ribosome. However, this system also suffered from competition with native initiator tRNAs. In addition, the detection was performed using the incorporated radioactive label rather than incorporated fluorophore.

Previously, various elongator tRNAs (wild-type and suppressors) were used to incorporate fluorophores into proteins during in vitro translation. For example, wild- type Cys-tRNA$^{Cys}$ was used for site-specific incorporation of BODIPY-FL into proteins with moderate labeling efficiency (2.5–9.7%) [33]. Suppressor elongator tRNAs carrying fluorophores provided much better incorporation efficiency ranging from 20% to as high as 80% [34–39].

In addition to BODIPY-FL-Met-tRNA$^{fMet}$, a system for fluorescent labeling based on BODIPY-FL-Lys tRNA$^{Lys}$ was developed previously [22] and is currently available from Promega Corp. under the trade name FluoroTect-Green$_{Lys}$. However, this system is also based on the naturally occurring tRNAs and therefore suffers from the competition with unlabeled tRNAs and consequent label dilution. In addition, the labeling with the BODIPY-FL-Lys-tRNA$^{Lys}$ is highly dependent on the lysine content in the translated protein. In contrast, the amber-1 labeling system offers a higher labeling efficiency and potentially a method of eliminating the variability due to protein composition.

In addition to higher efficiency, the amber-1 system eliminates one of the drawbacks of the *E. coli* S30 translation system—generation of truncated labeled protein products from internal methionine codons serving as false initiation signals. In the amber-1 system, unlike in the case of normal AUG, any internal TAG codons must be out of reading frame to initiate the synthesis. Should this occur, the truncated protein products generated would be extremely short and would not interfere with analysis of the target protein.

In general, substitution of radioisotope by fluorescence is highly desirable because it eliminates special handling, disposal, and regulatory requirements associated with its use. In addition, fluorescence offers much faster analysis, high sensitivity, and the possibility to use the same gel for additional downstream procedures such as a Western blot. It also enables a number of potential applications for protein analysis which utilize fluorescence, such as capillary electrophoresis, fluorescence polarization, and fluorescence resonance energy transfer.

The cause of the nonquantitative labeling observed with the amber-1 system (e.g., variability of labeling from 27 to 67%) needs to be further explored. However, it is likely that a major causal factor is label dilution due to the utilization of fma-tRNA to initiate protein synthesis with nonlabeled methionine. This would be possible if there are significant levels of deacylated fma-tRNA present to allow the *E. coli* enzymes and factors present in the T7 S30 to enzymatically charge the fma-tRNA and to be initiated with normal unlabeled amino acid. One possible way to improve the specific labeling would be to use fma-tRNA mutants such as $T_1$ or G72-G73 known to exhibit ineffective formulation but normal ribosome binding [40]. Another possible way would be to use a mutant initiator tRNA with a four or five base anticodon loop similar to the recently described elongator tRNAs [35,37,38,41].

Currently the use of amber-1 labeling is limited to the S30 *E. coli* translation system. RajBhandary and coworkers [42] demonstrated overexpression of human suppressor initiator tRNA in yeast. It has also been shown that protein synthesis in mammalian cells can be initiated with amino acids other than methionine [43].

It is therefore possible that such human initiator suppressor tRNA after chemical aminoacylation procedures could be used for nascent protein labeling in eukaryotic translation systems such as rabbit reticulocyte lysate and wheat germ extract.

## Acknowledgments

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